Infection and Drug Resistance

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ORIGINAL RESEARCH

Prevalence of Quorum Sensing and Virulence Factor Genes Among *Pseudomonas aeruginosa* Isolated from Patients Suffering from Different Infections and Their Association with Antimicrobial Resistance

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Purpose: Antimicrobial resistance and virulence genes play important roles in increasing the severity of *Pseudomonas aeruginosa* infections, especially in hospitalized patients with high antibiotic pressure. Most genes that encode *Pseudomonas aeruginosa* virulence factors are controlled and regulated by the quorum sensing (QS) system. The aim of this study was to investigate the frequency of some virulence genes (*rhlR, rhlI, lasR, lasI, lasB, toxA, aprA, algD, ExoS, and plcH* genes) and their association with antibiotic resistance.

Methods: Antimicrobial susceptibility was determined by Kirby–Bauer agar disk diffusion method. A total of 125 clinical isolates of *P. aeruginosa* were tested for some virulence genes using polymerase chain reaction (PCR).

Results: The highest resistance was observed against cefepime (92.8%). Multi-drug resistant (MDR) *P. aeruginosa* represented 63.2% of total isolates with high distribution among wound isolates (21/79, 26.3% of MDR isolates). *LasB* was the most prevalent virulence gene among the tested isolates (89.6%) followed by *aprA* (85.6%), *exoS* (84%), *algD* (80%), *toxA* (76.8%), and *plcH* (75.2). Furthermore, a significant association ($P < 0.05$) among most of the tested virulence genes and MDR isolates was found. The presence of more than 5 virulence genes was highly observed among wound infections, otitis media, and respiratory tract infection isolates.

Conclusion: The complex association of virulence genes including QS system regulating genes with antibiotic resistance indicates the importance of the tested factors in the progression of infections, which is considered a great challenge for the health-care team with the need for specific studies for each area having different antibiotic resistance profiles and the development of effective treatment strategies such as anti-virulent and quorum sensing inhibiting drugs against *P. aeruginosa* infections.

Keywords: *Pseudomonas aeruginosa*, quorum sensing, virulence genes, antibiotic resistance

Introduction

P. aeruginosa is one of the most life-threatening and resistant bacteria according to the World Health Organization (WHO), for which new antibiotics are urgently needed.[1] *P. aeruginosa* is also one of the most prevalent nosocomial pathogens worldwide.[2] It affects people who are susceptible, such as those with postoperative immune suppression or immunosuppressed patients.[3] Since this organism exhibits virtually all known mechanisms of antibiotic resistance, nosocomial infections caused by it are frequently challenging to be cured. These resistance mechanisms frequently occur in combination, conferring multi-resistant phenotypes.[2]

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Infections caused by *P. aeruginosa* include urinary tract infections, bacteremia, cystic fibrosis, lung infections, wound infections, especially of thermal burns, surgical wound infections, and otitis media.[4]

It is well recognized that *P. aeruginosa* infections significantly increase the risk of morbidity and mortality because of the organism's propensity to express a wide range of virulence factors, adapt readily to environmental changes, acquire antibiotic resistance[5] and producing agents such as pyocyanin, hemolysin, gelatinase, and biofilms that cause tissue injury and shield *P. aeruginosa* from immune system detection and antibiotic action.[6,7] Additional virulence factors that *P. aeruginosa* can produce include lipases, lecithinase, DNase, and proteases. Different mechanisms of *P. aeruginosa* resistance result in the emergence of isolates that are pan-drug-resistant (PDR) or multi-drug resistant (MDR).[8] The pathogen exhibits a variety of these mechanisms such as multidrug efflux pump, β-lactamases, and aminoglycoside modifying enzymes[9] that increase the chance of developing multidrug resistant strains representing a significant threat to health-care facilities and created increasing immense clinical global problem.[10] Bacterial resistance syndrome is a property of *P. aeruginosa*, as this strain possesses almost all known antimicrobial resistance mechanisms.[11] Additionally, infections brought on by isolates of the multi-drug-resistant (MDR) *P. aeruginosa* are linked to longer hospital stays, higher expenses, and higher rates of morbidity and mortality.[12]

In recent years, it has been discovered that *P. aeruginosa* can produce biofilm and many virulence factors under the regulation of quorum sensing (QS) mechanism that is a cell-to-cell communication process. It was found that QS systems are 2 systems named as Las and rhl system depending on N-acyl homoserine lactone (AHL) signal molecules (also named as autoinducers). Las system is comprised of the transcriptional regulatory protein LasR, its cognate autoinducer molecule N-(3-oxododecanoyl) homoserine lactone (3OC12-HSL) and the AHL synthase LasI, while Rhl system consists of RhlR, its cognate autoinducer molecule N-butyryl homoserine lactone (C4-HSL) and the AHL synthase RhlI. Autoinducers should reach a critical threshold concentration to bind its cognate transcriptional regulatory protein. Then, the autoinducer-transcriptional regulatory protein complex activates the expression of many target genes, along with the AHL synthase that results in forming a positive feedback loop. *Las* and *rhl* systems are two separate QS systems but related with each other by a hierarchical manner, with the dominance of *las* system over the *rhl* system.[13]

Numerous virulence factors present in *P. aeruginosa* may be crucial to the pathogenicity of this bacteria. It can produce many extracellular enzymes and toxins that can disrupt host cell membranes and can impair phagocytosis and host immune response. Toxin A (*toxA*) that can suppress protein synthesis and affect macrophage action, alkaline protease (*aprA*) can interfere with fibrin formation and inactivate host defense proteins, elastase that is able to split immunoglobulins and complement ingredients and impair neutrophil activity, and exoenzymes that are the primary virulence factors (S, U, T, *exoS, exoU*, and *exoT*) affecting protein synthesis. The breakdown of phospholipids in pulmonary surfactants may also be facilitated by two phospholipase C enzymes that are encoded by *plcH* (hemolytic phospholipase C) and *plcN* (non-hemolytic phospholipase C) which act together sequentially. *plcH* promote disruption of erythrocyte membranes exposing the inner leaflets followed by hydrolyzing phosphatidylserine in the inner leaflets by *plcN*.[14,15]

The present study was designed to study antimicrobial resistance patterns, identify Quorum sensing and virulence genes in the isolated strains of *P. aeruginosa*, and to study the association of QS genes and virulence genes with different types of infections and antibiotic resistance.

Materials and Methods

Bacterial Strains

In the current study, 125 *P. aeruginosa* strains were isolated from 350 different clinical specimens from patients admitted to the Minia University Hospital suffering from different infections (urinary tract infections 50 samples, respiratory tract infection, 87 sputum samples, otitis media, 83 ear discharge samples, wound infections; 85 samples and gastroenteritis; 45 samples). All clinical samples were obtained as part of the routine hospital laboratory procedures and labeled with the source and patient information. Ethical clearance for the study was granted by the ethics committee, faculty of Pharmacy, Minia University (No. HV16/2020). *P. aeruginosa* strains were confirmed using traditional microbiological methods and biochemical tests (culture on Cetrimide agar and Oxidase test). Biofilm formation was tested using the tissue culture plate method (TCP).[16]

The gold-standard technique for biofilm identification is the tissue culture plate (TCP) assay, which was first published by Christensen et al in 1995. In 10 mL of TSB with 1% glucose, isolates from freshly prepared agar plates were injected. Broths were incubated for 24 hours at 37°C. Then, fresh medium was added, and the cultures were diluted 1:100.

200 L of the diluted cultures were placed in each of the 96 wells of sterile flat-bottom tissue culture plates. For the purpose of testing media sterility and non-specific binding, only sterile broths were offered as a blank. Likewise, control organisms were diluted and incubated as well. The tissue culture plates contained the three controls as well as blanks.

The culture plates underwent a 24-hour incubation period at 37°C. The wells were cleaned four times with 0.2 mL of phosphate buffer saline (pH 7.2) before being let to dry naturally. The wells were next stained for 30 minutes at room temperature with 200 L of 0.1% crystal violet. To get rid of the excess color, the plates were rinsed with distilled water and then left to dry. The use of 200 L of 95% ethanol helped to dissolve the adherent stain. A micro ELISA auto reader operating at a wavelength of 630 nm was used to measure the optical densities (OD) of stained adherent biofilm. Three times of the experiment were carried out in duplicate. All test results were computed, and the average OD values of the sterile medium was subtracted. Non-biofilm producers were judged to have ODs below 0.120, moderate biofilm producers to have ODs between 0.120 and 0.240, and more than 0.240 as strong biofilm producers.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of *P. aeruginosa* strains was performed determined by the Kirby–Bauer disc diffusion method using Muller-Hinton agar (HiMedia, India) according to Clinical & Laboratory Standards Institute guidelines (CLSI).[17] The antibiotics evaluated were ciprofloxacin (CIP, 5 µg), amikacin (AK, 30 µg), cefepime (CPM, 30 µg), norfloxacin (NX, 10 µg), imipenem (IPM, 10 µg), gentamicin (GEN, 10 µg), tobramycin (TOB, 10 µg), aztreonam (ATM, 30 µg), piperacillin-tazobactam (PIT 100/10 µg), and colistin (CL, 10 µg). Zones of inhibition were recorded in mm. The susceptibility pattern was determined using the CLSI interpretation chart as susceptible (S), intermediate (I), or resistant (R).

Molecular Methods

A loop of bacterial cells was inoculated into Luria Bertani broth vials (Difco Laboratories, Detroit, Michigan, USA) containing 100 mg/mL ampicillin (Sigma, USA) and incubated at 37°C with shaking at 185 rpm for 16–18 h. DNA extraction was performed using an Extraction Kit (Qiagen kit, Germany) according to the manufacturer's instructions. Forward and reverse primers for 10 virulence genes were used. PCR amplifications were conducted to detect the genes listed in Table 1. The amplifications were conducted in a volume of 25 µL containing 12.5 µL of PCR Master mix (DreamTaq Green PCR master mix, Thermo scientific, USA), 1 µL of each primer (10 pM/µL) (forward and reverse), 1

Table 1 The Primers Used in This Study and Their Sequences

Primer Type	Primer Sequence (5'-3')	Amplicon Size (bp)	Annealing Temp.	Reference
lasB-F	GGAATGAACGAAGCGTTCTC	300	55	[59]
lasB-R	GGTCCAGTAGTAGCGGTTGG			
toxA-F	GGTAACCAGCTCAGCCACAT	352	50	[59]
toxA-R	TGATGTCCAGGTCATGCTTC			
aprA-F	ACCCTGTCCTATTCGTTCC	140	65	[59]
aprA-R	GATTGCAGCGACAACTTGG			
algD-F	ATGCGAATCAGCATCTTTGGT	1311	55	[59]
algD-R	CTACCAGCAGATGCCCTCGGC			
plcH-F	GAAGCCATGGGCTACTTCAA	307	55	[59]
plcH-R	AGAGTGACGAGGAGCGGTAG			
ExoS-F	CTTGAAGGGACTCGACAAGG	504	58	[60]
ExoS-R	TTCAGGTCCGCGTAGTGAAT			

(*Continued*)

Table 1 (Continued).

Primer Type	Primer Sequence (5'-3')	Amplicon Size (bp)	Annealing Temp.	Reference
LasI-F ***LasI-R***	CGCACATCTGGGAACTCA CGGCACGACGATCATCATCT	605	65	[61]
LasR-F ***LasR-R***	CTGTGGATGCTCAAGGACTAC AACTGGTCTTGCCGATGG	725	65	[61]
rhlI-F ***rhlI-R***	GTAGCGGGTTTGCGGATG CGGCATCAGGTCTTCATCG	155	65	[61]
rhlR-F ***rhlR-R***	GCCAGCGTCTTGTTCGG CGGTCTGCCTGAGCCATC	133	60	[61]

μL of template DNA, and nuclease-free water. The PCR conditions for amplification for all of the mentioned genomic regions were: initial denaturation for 5 min at 94° C, 35 cycles of denaturation for 60s at 94° C, annealing for 60s at 48° C, and extension for 90s at 72°C with a final extension at 72° C for 10 min.[18,19]

Following amplification, aliquots were removed from each reaction mixture and 100-bp phage ladders (BIOMATIK, USA) were examined by electrophoresis (70 V, 45 min) in gels composed of 1.5% (w/v) agarose (Promega, USA) in 1X TBE buffer (40 mM Tris, 20 mM boric acid, 1 mM EDTA, pH 8.3), and stained with ethidium bromide (5 g/100 mL). The gel bands were visualized under UV illumination using a gel image analysis system.

Statistical Analysis

To compare the frequencies obtained for virulence genes and antibiotic resistance, chi-square (X2) and Fisher's exact test were performed using SPSS version 17 statistical software (SPSS Inc., Chicago, IL). Correlations were determined using Spearman correlation (r) and Pearson's correlation coefficient (r2) in bivariate linear correlations ($P < 0.05$). The P-value was significant if it was ≤0.05.

Results

P. aeruginosa Isolation and Identification

A total of 125 *P. aeruginosa* isolates were isolated from a total of 350 samples including: urine (15/50), sputum (27/87), ear discharges (32/83), wound exudates (35/85), and stool (16/45). TCP method was used to detect the ability of *P. aeruginosa* isolates to develop biofilm. Out of 125 *P. aeruginosa* isolates, 78 (62.4%) isolates were detected as strong biofilm producers, 32 (25.6%) as moderate biofilm producers and 15 (12%) as weak or non-biofilm producers.

Antimicrobial Susceptibility

In this study, antimicrobial susceptibility profiles for 10 antibiotics, representing seven different classes, demonstrated high rates of antibiotic resistance. The resistance to ciprofloxacin, amikacin, cefepime, norfloxacin, imipenem, gentamicin, tobramycin, aztreonam, piperacillin-tazobactam, and colistin ranged from 12.8 to 92.8% (Figure 1). High incidence of antibiotic resistance of *P. aeruginosa* isolates against ciprofloxacin, cefepime, and colistin antibiotics was shown by strains isolated from urine, wound infection and sputum.

Table 2 shows the distribution of MDR isolates among different types of infections. The table showed that 63.2% of total *P. aeruginosa* isolates were MDR (as they were resistant to 3 or more of the tested antibiotics). In addition, it was found that multi-drug resistant isolates were highly distributed among sputum samples as 70.4% of sputum isolates were MDR, followed by urine isolates (66.7% of urine isolates) while 9 stool isolates only showed MDR pattern (56.3%). In addition, our results showed that the highest incidence of MDR isolates were obtained from wound infections, otitis media and respiratory tract infections (Table 3).

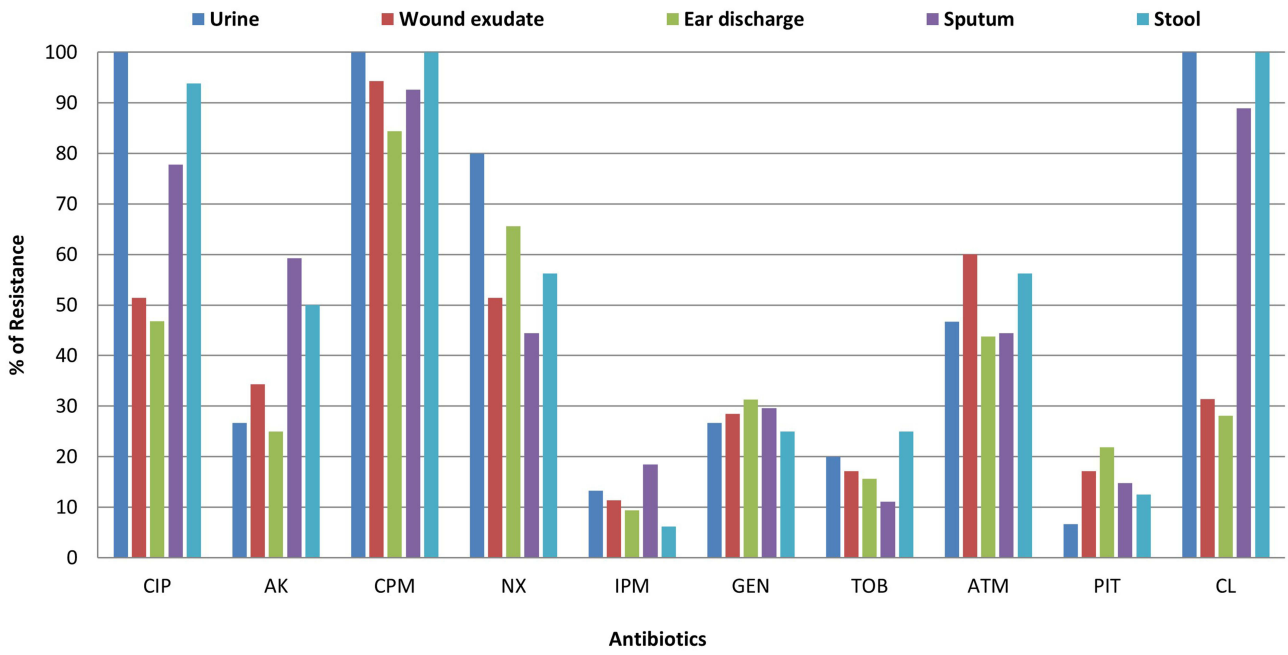

Figure 1 Antibiotic resistance profile of *P. aeruginosa* according to the type of sample.
Abbreviations: CIP, ciprofloxacin; AK, amikacin; CPM, cefepime; NX, norfloxacin; IPM, imipenem; GEN, gentamicin, TOB, tobramycin; ATM, aztreonam; PIT, piperacillin-tazobactam; CL, colistin.

Distribution of Virulence Genes Among Isolates of Different Type of Samples and MDR Isolates

The results indicated that 93.6% (117/125) of the isolates were positive for one or more of the tested virulence genes, while only 6.4% (8/125) were negative for all of these genes. The results were: 94.4% (118/125) of the isolates were positive for *rhlR*, 69.6% (87/125) were positive for *rhlI*, while 80% (100/125) were positive for *LasR*, and the remaining 81.6% (102/125) were positive for *lasI*. *lasB* was found to be the most common virulence gene detected in *P. aeruginosa* isolates (89.6%). Moreover, *aprA* (85.6%), *exoS* (84%), *algD* (80%), *toxA* (76.8%), and *plcH* (75.2%) genes that play important role in the pathogenesis of *P. aeruginosa* were identified in the evaluated isolates.

Regarding the distribution of virulence genes among isolates according to sample type, it was found that there is no significant correlation between virulence genes and the type of clinical sample, except for the *lasI* gene, which did exhibit a significant association, as shown in Table 4.

Table 2 Distribution of MDR Resistant *P. aeruginosa* Among Isolates Among Different Types of Infection

Sample Type	MDR		Non-MDR		P value
	No.	%*	No.	%*	
U	10	66.7	5	33.3	0.767
W	21	60	14	40	0.644
ER	20	62.5	12	37.5	0.924
SP	19	70.4	8	29.6	0.383
ST	9	56.3	7	43.7	0.537
Total	79	63.2**	46	36.8**	

Notes: Type of clinical specimen; U: urine specimen; W: wound exudate; ER: ear discharge; SP: sputum; ST: stool. **P. aeruginosa* isolates related to the total number of isolates from the same source. **The total number of *P. aeruginosa* isolates.

Table 3 Prevalence of MDR Isolates Among the Different Types of Infections

Type of Samples	MDR Isolates (N=79)	
	No.	%*
Urine	10	12.6
Wound	21	26.5
Ear discharge	20	25.3
Sputum	19	24.05
Stool	9	11.3

Note: *Percents were correlated to the total number of MDR isolates.
Abbreviation: MDR, Multi-drug resistant.

Table 4 Distribution of Quorum Sensing and Virulence Factors Regulating Genes in *P. aeruginosa* Isolated from Different Clinical Specimens

Tested Genes		Type of Sample %*					Total (%**)	P value
		U	W	ER	SP	ST		
QS genes	***rhlI***	46.7	74.3	71.9	70.4	75	69.6	0.355
	rhlR	100	100	93.8	92.6	81.3	94.4	0.078
	lasI	53.3	91.4	96.9	74.1	68.8	81.6	0.001***
	lasR	60	88.6	84.4	85.2	62.5	80	0.055
Virulence factors genes	***lasB***	86.7	88.6	90.6	92.6	87.5	89.6	0.969
	aprA	73.3	85.7	87.5	88.9	87.5	85.6	0.697
	exoS	80	82.9	90.6	85.2	75	84	0.690
	algD	66.7	80	84.4	81.5	81.3	80	0.717
	toxA	66.7	80	78.1	77.8	75	76.8	0.888
	plcH	60	77.1	75	81.5	75	75.2	0.644

Notes: **P. aeruginosa* isolates related to the total number of isolates from the same source. **The total number of *P. aeruginosa* isolates, ***P-value was significant if it was ≤0.05.
Abbreviation: QS genes, Quorum sensing genes.

A significant correlation between the distribution of the tested virulence genes and MDR resistance in *P. aeruginosa* isolates, especially QS genes (*rhlI, rhlR* and *LasR), LasB, aprA* and *exoS* as shown in Table 5.

Distribution of Biofilm Producers and Non-Producers Among MDR Isolates and Their Resistance Pattern Against Different Antimicrobial Agents

Most of MDR isolates were biofilm producers as biofilm acts as a barrier for the diffusion of antibiotics (Figure 2). Resistance pattern of biofilm and non-biofilm producing organisms showed that all producing *P. aeruginosa* were cefepime resistant, followed by ciprofloxacin (73.6%). Imipenem showed the highest activity against biofilm producing isolates. On the other hand, the highest activity against biofilm non-producing isolates was shown by tobramycin and imipenem (Table 6).

Table 5 Distribution of Virulence Factor Genes Among MDR and Non-MDR *P. aeruginosa* Isolates

		MDR		Non-MDR		P value
		No.	%*	No.	%**	
QS genes	***rhlI***	65	82.3	22	47.8	<0.001***
	rhlR	79	100	39	84.9	<0.001***
	lasI	70	88.6	32	69.6	0.008***
	lasR	74	93.7	26	56.5	<0.001***
Virulence factors genes	***lasB***	78	98.7	34	73.9	<0.001***
	aprA	76	96.2	31	67.4	<0.001***
	exoS	73	92.4	32	69.6	0.001***
	algD	69	87.3	31	67.4	0.007***
	toxA	68	86.1	28	60.9	0.001***
	plcH	67	84.8	27	58.7	0.001***

Notes: *The total number of MDR *P. aeruginosa* isolates (79). **The total number of Non-MDR *P. aeruginosa* isolates (46), ***P-value was significant if it was ≤0.05.

Distribution of QS and Virulence Genes Among *P. aeruginosa* Isolated from Different Types of Infection and Resistance Profile of Highly Virulent Isolates

Distribution of the tested virulence genes was observed among strains isolated from wound, ear discharge and sputum samples. Also, high distribution of *lasB* gene encoding elastase enzymes and *aprA* genes encoding alkaline phosphatase were found among sputum isolates showing their important effect on *P. aeruginosa* respiratory tract infections. It was noticed that

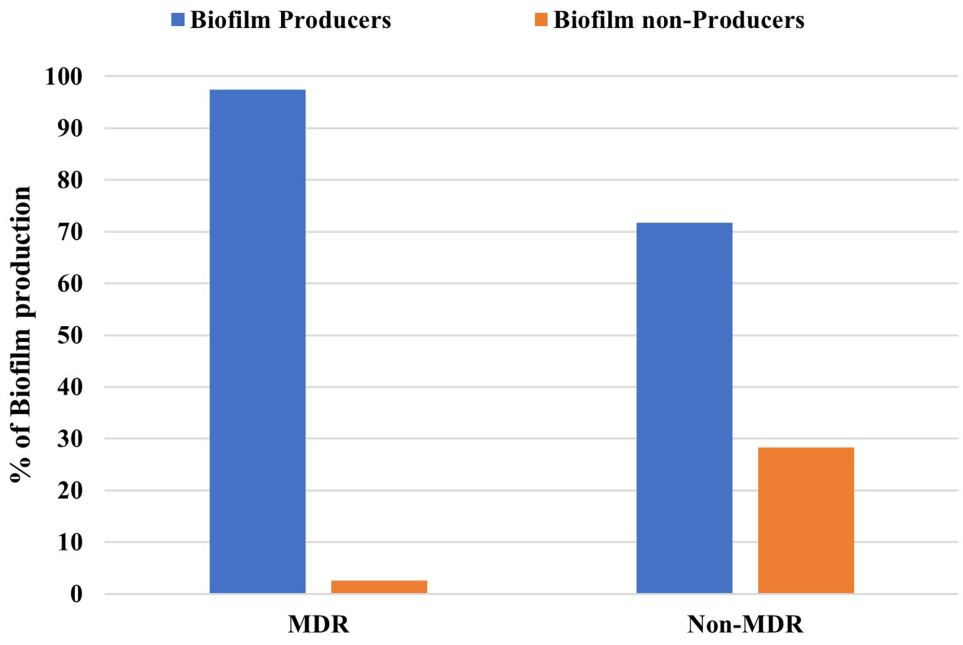

Figure 2 Correlation between biofilm formation and type of resistant *P. aeruginosa* isolates (P > 0.001).
Abbreviation: MDR, Multi-drug resistant.

Table 6 Resistance Pattern of *P. aeruginosa* Among Biofilm Producers and Non-Producer

Antimicrobial Agent	Biofilm Producers No. (%)*	Biofilm Non Producers No. (%)**	P value
Ciprofloxacin	81 (73.6)	3 (20)	<0.001***
Amikacin	47 (42.7)	1 (6.7)	<0.001***
Cefepime	110 (100)	6 (40)	<0.001***
Norfloxacin	70 (63.6)	2 (13.3)	<0.001***
Imipenem	15 (13.6)	0 (0)	0.127
Gentamicin	34 (30.9)	2 (13.3)	<0.001***
Tobramycin	21 (19.1)	0 (0)	0.179
Aztreonam	60 (54.5)	3 (29)	0.012***
Piperacillin-tazobactam	19 (17.3)	1 (6.7)	0.089
Colistin	73 (66.4)	2 (13.3)	<0.001***

Notes: *Percents were correlated to total biofilm producers (110). **Percents were related to total biofilm non producers (15). ***P-value was significant if it was ≤0.05.

strains isolated from sputum samples were positive for at least 3 virulence genes and maximum for 10 virulence genes, which indicates that strains able to cause respiratory infections are more virulent and highly resistant to antimicrobials. Our results showed also that 20% of total isolates were positive for 6 virulence genes, followed by the presence of 8 virulence genes in 16.8% of isolates especially those were isolated from ear discharge and sputum samples (Table 7).

Table 7 Distribution of Virulence Genes Among *P. aeruginosa* Clinical Isolates Collected from Different Sources

No. of Virulence Genes Found in Each Isolate	Type of Samples %*					Total (n=125) N (%**)	P value
	Urine	Wound Exudate	Ear Discharge	Sputum	Stool		
0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.893
1	0 (0)	1 (2.9)	0 (0)	0 (0)	1 (6.3)	2 (1.6)	
2	1 (6.7)	1 (2.9)	1 (3.1)	0 (0)	2 (12.5)	5 (4)	
3	2 (13.3)	2 (5.7)	3 (9.4)	1 (3.7)	1 (6.3)	9 (7.2)	
4	2 (13.3)	1 (2.9)	2 (6.3)	1 (3.7)	2 (12.5)	8 (6.4)	
5	4 (26.7)	5 (14.3)	2 (6.3)	4 (14.8)	2 (12.5)	17 (13.6)	
6	3 (20)	6 (17.1)	8 (25)	5 (18.5)	3 (18.8)	25 (20)	
7	2 (13.3)	4 (11.4)	4 (12.5)	5 (18.5)	1 (6.3)	16 (12.8)	
8	1 (6.7)	7 (20)	4 (12.5)	6 (22.2)	3 (18.8)	21 (16.8)	
9	0 (0)	5 (14.3)	6 (18.8)	3 (11.1)	1 (6.3)	15 (12)	
10	0 (0)	3 (8.6)	2 (6.3)	2 (7.4)	0 (0)	7 (5.6)	

Notes: *Percentages were correlated to the total number of *P. aeruginosa* isolates from each type of sample. **Percentages were correlated to the total number of *P. aeruginosa* isolates. P-value was significant if it was ≤0.05.

Resistance Profile of Highly Virulent *P. aeruginosa* Isolates Contain More Than 6 Genes Including QS Sensing Genes

Table 8 shows that all isolates (biofilm producers) harbor both lasI/or R and *rhlI*/or R except 2 isolates were positive for *rhl* system only (Group 2). These 2 isolates were positive for all virulence genes except *exoS* gene and resistant for 3 antibiotics. Furthermore, there were 3 isolates positive for Las system only (Group 3), positive for all virulence genes and resistant for 5 antibiotics, which indicates that Las system plays an important role in regulating virulence factors. It was found that all isolates positive for all QS genes were positive for *lasB* and *algD* genes that indicates the important impact of QS genes in regulating both genes. In addition, these isolates were resistant to 6 to 8 antibiotics. Previous results in this study indicated the impact of QS genes on regulating different virulence factors, biofilm production, which lead to tissue destruction and impairment for the immune response and the emergence of non-responsive cells and the development of antibiotic resistance. Table 9 shows significant association ($P < 0.001$) between groups containing different numbers of virulence genes and number of antibiotics showing low activity against the tested strains due to their resistance. In addition, strong positive correlation ($r = 0.815$, $P < 0.001$) between number of virulence genes and resistance to antibiotics (Table 10).

Discussion

It is well known that *P. aeruginosa* infections are associated with high morbidity and mortality due to its ability of adapting their environmental changes, to express many virulence factors and to acquire antibiotic resistance. In this study, 63.2% of total isolates were MDR. In addition, 92.8% of the isolates exhibited resistance to cefepime. A similar study from Iran reported approximately 56% resistance to cefotaxime in *P. aeruginosa* isolates from 600 isolates of *P. aeruginosa* that were cultured from patients at two hospitals in Tehran.[20,21] Based on the results, 16% of the isolates exhibited resistance to piperacillin-tazobactam, which is higher than other results obtained from study by Khan in Makkah and Jeddah (4.9%).[22] The highest activity against the tested isolates was shown by imipenem (12.8%). Similar results showing the highest activity of imipenem and piperacillin-tazobactam against MDR *P. aeruginosa* were reported by other studies (6% and 28%, respectively).[23,24] In the present study, 73% of *P. aeruginosa* isolates exhibited sensitivity to aminoglycoside antibiotics, gentamicin, tobramycin, and amikacin. The results from this study regarding gentamicin are in agreement with those from Shahcheraghi et al[23](69%), and Zahra et al[25] (77%), but differed from those of Nakhael et al[24] (45.31%). Moreover, Tambekar et al[26] and Ehinmidu[27] reported a high susceptibility to gentamicin. Low susceptibility to gentamicin was reported in two studies (32.2%, 33%, respectively) in 2014 by Fazeli et al[15] and Nwanze et al.[28] Also, our results showed that ciprofloxacin inhibited the growth of 32.8% of the isolates. Data from the present study showed a difference with those obtained previously by Saeidi et al,[29] who reported that ciprofloxacin showed inhibitory activity against 85% of isolates. Akingbade et al[30] reported that *P. aeruginosa* strains have moderate resistance to ciprofloxacin (43%), which disagrees with the results of this study. Ilham et al[31] and Nwanze et al[28] also reported moderate and high resistance to ciprofloxacin.[15,28,31] The infection process is aided by a number of *P. aeruginosa* regulatory and virulence factors. Understanding the pathogenesis of this opportunistic disease will be enhanced by the knowledge gained from studying these virulence factors. Moreover, it will help to define new targets to improve therapeutic efficacy and regimens. In the present study, 10 virulence factors, including *lasB, aprA, toxA, algD, plcH*, and *exoS*, were detected using PCR. Most of the virulence factor genes were detected in most of isolates. In addition, quorum sensing system was mostly observed among most of isolates. The Las system regulates the production of some virulence factors elastase, exotoxin A, and alkaline protease. In addition, Las system positively regulates *rhl* system. The *rhl* system induces the regulation of some virulence factors such as alkaline protease, biofilm formation by enhancing the biosynthesis of *pel* polysaccharide, elastase, rhamnolipid, pyocyanin, and HCN.

It was found that biofilm non-producing isolates showed no resistance against either imipenem and tobramycin while biofilm producing isolates showed resistance to imipenem and tobramycin. These results agreed with that reported by Bagge et al,[32] who reported that imipenem cause changes in biofilm structure in biofilm-producing isolates due to its ability to increase alginate production. Also, our results showed that non-biofilm producing isolates were completely

Table 8 Resistance Pattern of Highly Virulent Biofilm Producing *P. aeruginosa* Harboring ≥6 Virulence Genes

No. of Virulence Genes	No. of Isolates	QS Genes				Virulence Genes						Antibiotics									
		lasI	*lasR*	*rhlI*	*rhlR*	*lasB*	*aprA*	*algD*	*toxA*	*exoS*	*plcH*	CIP	AK	CPM	NX	IPM	GEN	TOB	ATM	PIT	CL
Group 1 (6 genes)	**7**	+	+	+		+		+		+		+		+					+		+
	6	+		+	+		+		+	+		+	+	+	+						
	5		+		+	+		+	+		+	+		+			+				+
	3	+	+		+		+			+	+			+	+						+
	2		+	+		+	+	+	+			+						+	+		
	2	+		+	+	+			+		+		+			+					+
Group 2 (7 genes)	**5**		+	+	+	+	+	+		+		+		+			+				
	4	+		+		+		+	+	+	+			+	+						+
	4	+	+		+	+	+	+		+		+		+					+		
	2			+	+	+	+	+	+		+		+	+			+				
	1	+	+		+	+	+			+	+	+				+					+
Group 3 (8 genes)	**6**	+	+	+	+	+	+	+		+		+		+	+				+		+
	5	+	+	+	+	+	+	+			+	+		+	+					+	
	3	+		+		+	+	+	+	+	+		+	+	+		+		+		
	3	+	+			+	+	+	+	+	+	+	+					+	+		+
	2		+	+	+	+		+	+	+	+		+	+		+	+		+		+
	1	+	+	+	+	+	+	+		+	+	+		+	+			+	+		+
	1	+	+	+	+	+	+	+	+	+		+		+		+		+	+	+	
Group 4 (9 genes)	**5**	+	+	+	+	+	+	+		+	+	+	+	+	+		+		+		
	5	+	+	+	+	+	+	+	+		+	+		+	+		+	+	+		+
	3	+	+		+	+	+	+	+	+	+	+		+	+	+		+	+		+
	2	+	+	+		+	+	+	+	+	+	+	+	+		+		+	+	+	
	2	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+		+	+	
Group 5 (10 genes)	**7**	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+		+	+	+

Abbreviations: CIP, ciprofloxacin; AK, amikacin; CPM, cefepime; NX, norfloxacin; IPM, imipenem; GEN, gentamicin; TOB, tobramycin; ATM, aztreonam; PIT, piperacillin-tazobactam; CL, colistin.

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Table 9 Correlation Between Different Groups of Virulent Genes of Highly Virulent Biofilm Producing *P. aeruginosa* and Number of Resistant Antibiotics

	Group I	Group II	Group III	Group IV	Group V	P value
	N=25	N=16	N=21	N=17	N=7	
No of resistant antibiotics	3.7±0.5	3±0	5±0.7	6.8±0.6	8±0	<0.001*

Notes: Group I: strains which were positive for 6 genes, Group II: strains which were positive for 7 genes, Group III: strains which were positive for 8 genes, Group IV: strains which were positive for 9 genes and Group V: strains which were positive for all tested genes. *P-value was significant if it was ≤0.05.

Table 10 Correlation Between Number of Genes and Number of Antibiotics

	Number of Antibiotics	
	r	P value
Number of genes	0.815	<0.001*

Notes: Spearman correlation (r), *P-value was significant if it was ≤ 0.05.

susceptible to tobramycin. Susceptibility of pseudomonas aeruginosa to tobramycin was reported by Koeppen et al[33] who reported that tobramycin reduced several outer membrane vesicles (OMVs) associated virulence determinants.

Our results showed that all MDR isolates were positive for *rhlR*. Regarding previous results obtained by previous studies, one can conclude that the resistance of *P. aeruginosa* strains to various antibiotics is relatively high. Resistance profile differs according to the time, the type of infection and if samples obtained from inpatient or outpatients. However, the results of these studies differ according to the time and location of isolation. In addition, the frequency of four QS genes (*lasI, lasR, rhlI, and rhlR*) in *P. aeruginosa* strains was analyzed in the present study. Different *P. aeruginosa* virulence factors are controlled by a gene system that is referred to as the QS system. In our study, results showed that the highest gene frequency was associated with rhlR in 118 strains (94.4%). The frequency of *rhlI, lasR*, and *lasI* genes was 69.6%, 80%, and 81.6%, respectively, in all tested isolates. By screening of 54 *P. aeruginosa* strains with simultaneous resistance to three antibiotics, 49 strains were found to be positive for the *rhlR* QS gene. A significant correlation between strains resistant to three antibiotics and *rhlR* gene distribution was observed. In comparison to other studies, Kadhim et al[34] showed that the frequencies of *lasR, lasI, rhlR*, and *laslI* QS genes were 5%, 78.3%, 65%, and 43.3%, respectively. Aghamollaei et al[35] reported the frequency of the *LasI* QS gene to be 48.5%. In a study performed by Senturk et al,[36] four isolates were found to be positive for *lasR, lasI, rhlR*, and *rhlI* genes. One of the isolates lacked the *lasR* gene, and one isolate was negative for *lasR, lasI*, and *rhlR*.

Production of extracellular enzymes that break down QS coding genes may be the cause of QS gene deficiency.[37] QS coding gene mutations may also contribute to variations in QS gene expression. The function of the *LasR* and *RhlR* genes is compromised, according to Bjarnsholt et al[37] due to increased mutations in QS genes. The expression of QS genes may be compromised by the presence of many *P. aeruginosa* strains at the infection site.[36]

Additional studies are required to determine the reason for these variations in QS gene expression and how they relate to the drug resistance of different strains of *P. aeruginosa*. According to the results of the present study, the highest frequencies were shown by *rhlR* (94.4%) and *lasI* (81.6%) indicating that the QS system plays an important role in the pathogenicity of *P. aeruginosa*. Moreover, these genes were primarily observed in clinical isolates that are MDR. Therefore, further investigations for determining a relationship between these genes and drug resistance are required. Many studies reported the important role of QS system in regulating virulence genes. LasI produces 3-oxo-C12-HSL that is able to activate LasR (cytoplasmic receptor), which has the ability to regulate the expression of genes controlling exotoxin-A production, proteases, biofilm formation, hemolysins, elastases.[38,39] On the other hand, RhlI produces the C4-

HSL, which associates with its cognate receptor RhlR resulting in the expression of many virulence genes controlling the production of pyocyanin, hydrogen cyanide, siderophores, elastases, alkaline protease and regulating bacterial motility[39,40] The prevalence of resistance to several antibiotics is the major problem linked to infections formed by biofilm producer bacteria.[41] Extracellular matrix production is a sign of a formed biofilm and works as a barrier to antibiotics, decreasing sensitivity to them.[42] Biofilm is known to prevent the distribution of antibiotics, makes it more challenging to treat patients with pseudomonal infections, which are confirmed by many researchers.[41,43]

Overall, *lasB* (89.6%) was the most detected virulence genes among *P. aeruginosa* isolates especially among sputum isolates due to its importance in lung infections as it leads to the degradation of connective tissues in lungs by inducing tissue injury and hemorrhage through destroying elastin, collagen types III and IV, laminin, fibronectin, and vitronectin of host cells. In chronic infections, elastase B destroy many components of the immune defense mechanism such as tumor necrosis factor-α, interferon-γ and interleukin-2 resulting in the damage of host cells.[44] Regarding the previous destructive effects of elastase B, it was found that *las B* was the most common virulence genes among MDR which indicates its role in the emergence of non-responsive bacterial cells to antibiotics. *aprA* genes were found to be highly distributed in sputum isolates (88.9%). Moreover, *aprA* can destroy host proteins such as complement proteins and cytokines resulting in alveolar hemorrhage, necrosis of alveolar septal cells, and infiltration of mononuclear cells enhancing its survival in tissues.[45] A study performed by Mittal et al,[46] a high level of production of elastase, protease, phospholipase C and toxin A was reported among isolates obtained from urinary tract infections in comparison to burn, wound infections and acute pneumonia. Regarding previous results, levels of these virulence factors showed different patterns based on the infection site.

In the present study, a high level of *algD* was observed among the tested isolates (80%), *algD* gene, regulating alginate capsule production by *P. aeruginosa*, plays a significant role in chronic lung infections.[47,48] Its expression may be associated with biofilm formation that also affects the therapeutic activity of antibiotics by acting as a barrier for their direct effect on the organism. It was documented that *algD, algU*, and *rpoS* genes are up-regulated in biofilm producing *P. aeruginosa*.[49] *algD, exoS* genes were shown to be common among ear discharge samples, while the *toxA* gene was highly distributed among wound swabs' samples, which in agreement with that reported by Morin et al.[50]

Regarding phospholipase C enzymes (PLCs), *PlcH* hemolytic and *PlcN* nonhemolytic, it was reported that injecting mice with large dosages may result in vascular permeability, organ damage, and death by the action of *PlcH*.[51] In the present study, *plcH* gene was highly distributed among sputum isolates (81.5%) in comparison to other types of samples due to its important role in degrading pulmonary surfactants, destroying cytoplasmic membranes and inhibiting opsonin. Wargo et al[52] reported that inhibition of hemolytic phospholipase C protects lung function during infections. Unlike previous studies from Iran, the results confirmed the low presence of these genes in 38.8% of *P. aeruginosa* isolates.[15]

In this study, more than 82.3% of MDR isolates expressed all tested genes that indicates their role in the development of resistance against antibiotics due to their pathophysiological destructive effect for host cell and different components of immune system leading to the selection of non-responsive bacterial cells and increasing the bacterial cell ability to adapt their environment. Galdino et al[53] reported that targeting elastase B (metalloenzyme) with metal-based antibiotics can reduce the emergence of antibiotic resistance. On the other hand, co-existence of 3 to 10 virulence genes in association with high presentation of MDR activity against the tested antibiotics was shown by respiratory tract, otitis media and wound infections isolates, which is in agreement with results obtained from Moradali et al.[54] Anju et al[55] and Qin et al.[56]

A wide spread of antibiotic resistance especially among strains isolated from patients suffering from respiratory tract infections. Increasing resistance against ciprofloxacin, cefepime and colistin are of great concern. *P. aeruginosa* is a pathogen that can accommodate their environment, express several virulence factors and show multidrug resistance pattern making the treatment of infections caused by this bacterium difficult and a great challenge. *P. aeruginosa* isolates exhibit a great degree of variation in their pathogenicity. Association of virulence genes with the type of infection indicates the important role of these factors in the stabilization of infection and to resist host immune response, which was well expressed among strains isolated from respiratory tract infections. Our results showed that QS genes have an important role in increasing the virulence of pathogens in association with the increase of antibiotic resistance resulting in the spread of life-threatening infections that is considered as a great challenge facing the health-care team. Quorum

sensing inhibitors development is an essential need for controlling *P. aeruginosa* infections. Many researchers reported the activity of some compounds as anti- quorum sensing agents. It was found that some phytochemicals sharing a heterocyclic ring structure similar to AHL molecules have the ability to interact with QS receptors and have the ability to degrade signal receptors such as ajoene, allicin, curcumin, cinnamaldehyde, eugenol. In addition, quercetin can inhibit biofilm production and twitching motility.[57] Olaniyi et al.[58] Benzene ethanamine, 4-methoxy- and Cyclopentadecanone, 2-hydroxy- from *Psidium guajava* leaves showed inhibitory properties against QS proteins of *Salmonella Typhi*.

Conclusion

P. aeruginosa causing respiratory tract, otitis media and wound infections are much more virulent and highly resistant strains in comparison to those isolated from other sources. A significant correlation between the abundance of the tested virulence genes and MDR resistance in *P. aeruginosa* isolates. Studying antimicrobial susceptibility patterns and distribution of virulence genes may be helpful for developing effective treatment strategies such as anti-virulent and Quorum sensing inhibiting drugs against *P. aeruginosa* infections.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, prior approval (No. HV16/2020) by the ethical committee of Faculty of Pharmacy, Minia University.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

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Disclosure

The authors declare no conflicts of interest in this work.

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